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(54) Title: BINDING ASSAY EMPLOYING LABELLED REAGENT

(57) Abstract

A binding assay process for an analyte, in which process a capture binding agent having binding sites specific for the analyte and a developing binding material capable of binding with the bound analyte or with the binding sites on the capture binding agent occupied by the bound analyte or with the binding sites remaining unoccupied on the capture binding agent are used, employs the capture binding agent in an amount such that only an insignificant fraction of the analyte in the sample becomes bound to the capture binding agent, the capture binding agent preferably being present at high surface density on microspots. A label is used in the assay in relation to the developing binding material, the label being provided by microspheres having a size of less than 5 μm and carrying a marker, preferably fluorescent dye molecules contained within the microspheres. For determination of the concentration of the analyte in the sample the strength of the signal is representative of the fractional occupancy of the binding sites on the capture binding agent by the analyte and a comparison is made with a dose-response curve computed from standard samples. For detection of an analyte comprising a single-stranded DNA sequence the presence of the analyte is detected by the existence of a signal. A kit for the process comprises a solid support having the capture binding agent immobilised on it, a developing reagent comprising the developing binding material attached to the microspheres and, where concentrations are to be measured, standards having known amounts or concentrations of the analyte to be determined.

Binding assay employing labelled reagent

Field of the invention

The present invention relates to binding assays employing a labelled reagent. Binding assays include immunoassays for the determination of concentrations of antigens in liquid samples, and it is also possible to use the present invention for the determination or detection of other analytes in liquid samples, including DNA sequences.

The present invention has particular relevance to non-competitive sandwich assays, that is to say assays in which a liquid sample containing an antigen or other analyte to be assayed such as a hormone is contacted with a first binding agent (such as an antibody) having binding sites on its molecule specific for the analyte whereby a fraction of the binding sites on the first binding agent representative of the concentration of the analyte in the liquid sample are occupied by the analyte. The fractional occupancy of the binding sites is then determined by a back-titration technique involving the use of a second binding material which is capable of binding with the bound analyte or with the binding sites occupied by bound analyte but not with unoccupied binding sites. Conveniently, the first binding agent will be referred to hereinafter as the capture binding agent and the second binding material will be referred to hereinafter as the developing binding material.

Non-competitive assays are to be distinguished from competitive assays in which the back-titration technique involves the use of a developing binding material which competes with the analyte for the binding sites on the capture binding agent, for example a labelled version of the analyte or another material able to bind with the unoccupied binding sites on the capture binding agent, although the present invention can also be used in such assays. In each case the extent of binding of the developing binding

scarce and costly to produce. It also involves the use of various stratagems to maximise the total surface area of the solid support on which the capture antibody is deposited. For example, porous glass microspheres have been used as a solid support in sandwich assay systems, the pores greatly increasing the surface area available for antibody attachment.

Roger Ekins has previously argued, for example in WO-84/01031, WO-88/01058 and WO-89/01157, that this general perception is mistaken and that, in certain circumstances, assays which have an even greater sensitivity than that attainable under the conditions mentioned above can be developed when the unknown sample and standard samples containing the analyte are each contacted with such a small amount of the capture binding agent that only an insignificant fraction of the analyte becomes bound to the capture binding agent. (This insignificant fraction is usually less than 5% and ideally 1-2% or less of the total amount of the analyte in the sample, bearing in mind that errors in analyte determination unavoidably introduced into the measuring procedure elsewhere by limitations in the accuracy of sample and reagent manipulation, signal measurements, standardisation, temperature variation and the like are generally of the order of 10% of the analyte in the sample, although sometimes the binding of higher fractions of the analyte up to 10% or so may be tolerated when exact determination is less important.) Only when an insignificant fraction of the total amount of analyte becomes bound is the fractional occupancy F of the binding sites on the capture binding agent related to the concentration [A] of analyte in the sample (at thermodynamic equilibrium) by the equation

$$F = \frac{K[A]}{1 + K[A]}$$

where K is the affinity constant of the capture binding

support in the form of a microspot, non-competitive assay systems may be devised which are as rapid to perform as or even more rapid to perform than, and possess sensitivities comparable with or indeed greatly superior to, those of conventional sandwich systems relying upon comparatively large amounts of capture binding agent. This crucial finding, which contradicts currently accepted views on the design of high sensitivity assays and is totally unexpected, potentially forms the basis of development of a variety of superior miniaturized diagnostic devices possessing exceedingly high sensitivity whilst requiring only relatively short incubation and measurement times.

Of course, the microspheres can also be used for labelling purposes in a competitive assay system using similarly very small amounts of capture binding agent, but in such systems the limit on sensitivity may not in practice be the specific activity of the label, and corresponding or substantial increases in sensitivity due to the use of the microspheres would therefore not necessarily be achieved or even expected, although increases in rapidity can be expected.

According to the present invention there is provided a binding assay process in which the concentration of an analyte in a liquid sample is determined by comparison with a dose-response curve computed from standard samples, using a capture binding agent having binding sites specific for the analyte and a developing binding material capable of binding with the bound analyte or with the binding sites on the capture binding agent occupied by the bound analyte or with the binding sites remaining unoccupied on the capture binding agent, the capture binding agent being used in an amount such that only an insignificant fraction of the analyte in the sample becomes bound to the capture binding agent, and a label being used in the assay in relation to the developing binding material whereby the strength of the signal associated with the label is representative of the

chemilumienescent labels will in general) be present on the surface of the spheres. Each microsphere desirably contains large numbers of fluorescent dye molecules as labels, for example up to 10 million in 1 μm diameter spheres with smaller numbers in smaller spheres (e.g. 100 or 1,000 to 100,000 or 1 million) down to about 10 in very small spheres. The fluorescent dye molecules may be selected to provide fluorescence of the appropriate colour range (excitation and emission wavelength) compatible with standard filter sets, for example yellow/green, orange or red, or customised filter sets may be used. Fluorescent dyes include coumarin, fluorescein, rhodamine and Texas Red. The fluorescent dye molecules may be ones having a prolonged fluorescent period such that the strength of the signal emitted by them can be determined by the known time-resolved fluorescence technique after background interference has decayed, for example lanthanide chelates and cryptates. Dyes which fluoresce only in non-aqueous media can be used. Preferred fluorescent dyes for use in the microspheres are oil-soluble dyes in order to facilitate their incorporation into the interior of the microspheres. Yellow/green, orange and red FluoSpheres, which are excited very efficiently at the 488, 568 and 647 nm krypton/argon mixed gas laser lines, respectively, are presently preferred.

In use as the label for the developing binding material, or for the capture binding agent and the developing binding material, in the assay systems of the invention the microspheres may have the developing binding material, or avidin which can be used as a "universal marker" reagent and bind all biotinylated binding material, or the capture binding agent as the case may be, physically adsorbed onto the surface of the spheres. More conveniently, however, appropriately surface-modified microspheres are selected and the developing binding material (eg. antibody) or capture binding agent (eg. antibody) is covalently bonded to them either directly or

binding agent on the microspot is desirably in the range 1,000 to 100,000 molecules/μm^2, preferably 10,000 to 50,000 molecules/μm^2 in the case of antibodies. For other binding agents the surface density may be within this range or above or below it but should preferably be as high as possible without sterically hindering binding of the analyte molecules. These microspots are used in conjunction with sample sizes of generally 1 ml or less, for example down to 50 or 100 μl or even less depending on the size of the microspot, the aim being to cover the microspot.

The microspot technique can be used to determine different analytes in the same or different liquid samples in a single operation by immobilising different capture binding agents on different microspots, for example 10 or more, e.g. up to 50 or more, on the same solid support and using different or identical developing binding materials labelled with the microspheres for the different binding assays. The labels (e.g. fluorescent dyes) associated with different binding assays and/or the techniques used to measure the signal strengths will be chosen to enable the results from the different assays to be differentiated. Techniques for this are known, for example from WO-88/01058.

To optimise the results achievable with the present invention a number of different features should be optimised, including the following:-

i) the fractional occupancy of the capture binding agent by the analyte,

ii) the size of the affinity constant of the capture binding agent for the analyte at the time when measurement occurs and, if the measurement is to be performed before equilibrium has been reached, the rate at which equilibrium is reached,

iii) the surface density of the capture binding agent on its support,

iv) the size of the microspots,

amount of capture binding agent (in units of 1/K, the x-axis) on the coated area. Clearly, as the area coated with capture binding agent (and hence its concentration) increases, the percentage of total analyte bound increases, but the signal/noise ratio falls. This effect is shown pictorially in Figure 2 of the accompanying drawings, where d is the diameter in mm of the area coated with capture binding agent, [Ab] is the concentration of binding agent assuming a surface density of 0.1/K per mm^2, and s/n is the signal/noise ratio expressed as a percentage of the value observed as the surface area approaches zero. This Figure likewise endeavours to show that, as the coated area increases, the amount of analyte bound also increases but the signal/noise ratio and hence the sensitivity fall.

However, although the signal/noise ratio R is highest when the binding agent concentration is less than 0.01/K, it is clear that the ratio may still be acceptably high when the amount of capture binding agent used equals or even exceeds 1/K. The upper limit to the amount of binding agent coated on the microspot area is preferably 10/K. This implies a ten-fold lower sensitivity than is achievable using a 1000-fold lower amount of binding agent and it should be emphasized that, although the invention is capable of yielding very high sensitivity, it is <u>also</u> applicable where lower sensitivity than the maximum attainable is acceptable.

For factor ii) it should be noted that, although at first sight it might appear to be better to use a capture binding agent with a low equilibrium value of K, it is in fact generally found that for high-sensitivity assays it is better to use binding agents having higher values of K at equilibrium, e.g. 10^{11} - 10^{12} or more litres/mole, and (if necessary or desired) to make the measurement before equilibrium has been reached so that at the moment of measurement only an insignificant fraction of the analyte

noise and reduce the signal/noise ratio.

For factor v) it should be noted that the support used will itself contribute to the noise level. If the level of background noise is a problem it may be preferable to use a black support rather than a white one, although this may decrease the signal level and some black supports have higher noise levels than others.

For factor vi) it is preferred to maximise the signal/noise ratio. Accordingly, the area from which the signal is measured is desirably small, preferably limited to the area of the microspot or a portion of it. Measuring the signal from a wider area beyond the microspot increases the noise level without increasing the signal level and thus decreases the signal/noise ratio. Hence it may be desirable to concentrate the illumination and to make measurements by means of a confocal microscope or other instrument achieving very precise illumination.

For factor vii) it is desirable that, after adsorption or covalent bonding of the developing binding material or capture binding agent to the microspheres has been carried out, the unreacted sites on the microspheres are blocked to avoid their non-specific binding to other biological molecules or the solid support for the capture binding agent. Blocking may be carried out with any non-interfering protein material. An albumin, particularly bovine serum albumin, is preferred. It has been found desirable to block not only with bovine serum albumin (BSA) or equivalent but also with a detergent such as TWEEN-20 or other non-ionic detergent. It is believed that there are some binding sites on the microspheres which are not blocked by BSA or other protein material alone. Microspheres blocked with BSA alone appear still to have binding sites which are capable of binding to the solid support, such as the plastic walls of the microtitre wells in which the assay is performed, or to other biological or non-biological

nucleotide sequences, which will differ from one another. The analyte may contain only one epitope for the capture binding agent or the epitope may be replicated on the analyte molecule. The polyclonal developing binding material (antibody) may react with a variety of epitopes on the analyte or the analyte capture binding agent complex, or a mixture of two or more monoclonal developing binding materials (antibodies) reacting with different epitopes may be used.

When used for nucleic acid (DNA) assays the DNA probe, a single-stranded nucleotide sequence, eg an oligonucleotide sequence of conventional or standard type, is attached as capture binding agent to a solid support and this recognises a corresponding single-standard DNA sequence constituting the analyte in a liquid sample and such sequences become bound so as to form a twin-stranded sequence. DNA probes consisting of oligonucleotide sequences are available commercially from a number of companies, e.g. Clontech Laboratories Inc., or they can be synthesised to order and/or modified (e.g. with biotin or digoxigenin) by commercial companies, e.g. British Biotechnology Products Ltd. The developing binding material may be either a labelled antibody which recognises the twin-stranded sequence as opposed to single-stranded sequences (see Figure 3 of the accompanying drawings) or another DNA sequence which recognises another part of the DNA sequence constituting the analyte and is labelled (see Figure 4 of the accompanying drawings), both these binding materials producing non-competitive assays. For competitive assays it is possible to use a labelled developing binding material recognising unoccupied sites of the capture binding agent, ie residual DNA probe not bound to analyte (see Figure 5 of the accompanying drawings). In each case the label is provided in accordance with the invention by the microspheres carrying a marker, preferably molecules of a fluorescent dye contained within the microspheres. In each of Figures 3-5 A represents a microspot, B the capture

or indirect chemical bonding.

According to a further embodiment of the invention there is provided a binding assay process for the detection of an analyte comprising single-stranded DNA sequence in a liquid sample, the process comprising contacting the sample in a non-competitive or competitive procedure with an immobilised capture binding agent which is a single-stranded oligonucleotide DNA probe capable of recognising analyte in the liquid sample and binding therewith, and with a labelled developing binding material which either is an antibody capable of recognising only twin-stranded DNA sequences formed from the probe and the analyte and of binding therewith or is an oligonucleotide DNA sequence capable of recognising and binding with either another part of the analyte or the residual probe, the developing binding material being labelled by means of microspheres having a size of less than 5 μm and carrying a marker, and, after the removal of unattached developing binding material, detecting the presence of the analyte by the existence or strength of a signal from the marker attached to developing binding material which has become bonded directly or indirectly to the immobilised capture binding agent.

Preferably, the marker is a fluorescent label, eg in the form of a large number (100 or more) of fluorescent dye molecules contained within microspheres having a size of 0.01 to 1 μm, eg 0.05 - 0.5 μm. It is preferred to use this technique in conjunction with the microspot technique already referred to, with the capture binding agent being immobilised as one or more microspots on a solid support at the surface densities and microspot sizes already mentioned and optionally different capture binding agents being immobilised on different microspots on the same support to enable a plurality of different DNA sequences to be detected or determined in a single operation using appropriately differentiated developing binding materials and signal detection or signal strength measurement techniques.

standards may also be provided as buffered solutions containing the analyte at known concentrations or in freeze dried form with instructions for appropriate reconstitution in solution form. The standards may be made up in hormone-free serum. There may be three or more standards, e.g. up to 12, of varying known analyte concentrations spanning the expected values in the unknown samples.

Preferably, the developing reagent contains the developing binding material adsorbed onto or covalently bonded to microspheres having a size of less than 5 μm and containing molecules of a fluorescent dye, and it is preferred that the solid support has the capture binding agent immobilised thereon in the form of one or more microspots of size less than 1 mm^2 and surface density at least 1000 molecules/ μm^2. Different capture binding agents may be immobilised on different microspots on the same solid support and a plurality of different developing reagents and different sets of standards may be provided so that a variety of different assays for different analytes may be performed using the same solid support in a single operation, simultaneously or sequentially.

The invention is further described in the following Examples, which illustrate the preparation of the labelled developing binding material (Examples 1-4) and processes and kits according to the invention (Examples 5-12).

In the Examples concentration percentages are by weight.

Example 1

Adsorption of Antibody or Avidin on Hydrophobic Sulfate-Microspheres

1) 0.5 ml of 2% solids suspension in pure water of surfactant-free sulphate-activated microspheres of polymer

Example 2

Covalent Coupling of Antibody or Avidin to Carboxylate-Modified Latex Microspheres by a one-step method

1) 0.5 ml of a 2% solids suspension in pure material of carboxylate-modified polymer latex microspheres containing fluorescent dye molecules (FluoSpheres from Molecular Probes Inc) and having a diameter of 0.09 μm was added dropwise to 0.5 ml of 0.015 M, pH 5 acetate buffer containing 2 mg of antibody or avidin as developing binding material. The suspension was incubated at room temperature for 15 min.

2) 4 mg of EDAC [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide] (Sigma Chemical Company) was added to the mixture and vortexed. The pH was adjusted to 6.5 ± 0.2 with dilute NaOH (agglomeration of the latex microspheres may be observed at this stage, but they can be redispersed by gentle sonication) and the reaction mixture was mixed gently overnight at 4°C.

3) The reaction mixture was centrifuged at 20,000 rpm for 30 min at 10°C. The supernatant was recovered for protein estimation.

4) The centrifuged pellet was dispersed in 1.0 ml of 0.1M phosphate buffer by sonication. After dispersion, the unoccupied sites on the microspheres were blocked by the addition of 1 ml of 2% (1% final) bovine serum albumin (BSA) and shaken for 2 hours at room temperature. The spheres were further blocked by the addition of 200μl of 5% Tween-20 (-0.5% final) and shaken for a further 1 hour at room temperature.

5) The preparation was centrifuged as above and the microspheres resuspended in 2 ml of 0.1M phosphate buffer.

6) Step 5 was repeated twice and, after the final

supernatant was kept for protein estimation.

9) The centrifuged pellet was resuspended in 1 ml of 0.1M ethanolamine in borate buffer, mixed gently for 30 min at room temperature and centrifuged as above.

10) The centrifuged pellet was resuspended in 1 ml of 1% BSA, mixed gently for 1 hour and centrifuged as above.

11) The centrifuged pellet was resuspended in 1 ml of 0.5% Tween-20, mixed gently for 1 hour and centrifuged as above.

12) The centrifuged pellet was resuspended in 1 ml of 0.02 M phosphate buffer, pH 7.4, and centrifuged as above.

13) The centrifuged pellet was resuspended in 1 ml of phosphate buffer containing 0.2% BSA and 0.01% of sodium azide and kept at 4°C.

Example 4a

Coupling of a Mixture of Antibody and Avidin to Microspheres by Adsorption or Covalent Linkage

The methodologies for the coupling of a mixture of antibody and avidin to microspheres by adsorption or covalent linkage were essentially the same as those described in Examples 1 to 3 above for the coupling of antibody or avidin to microspheres except that the antibody solution used for the reaction also contained a small amount of avidin.

Example 4b

Labelling of a Monoclonal Anti-TSH Antibody with Texas Red

1) 1 mg of monoclonal anti-TSH antibody was dissolved in 1 ml of carbonate buffer pH 9.

(0.1%) which was stored at 4°C.

Example 5

An Ultra-sensitive Sandwich Two-step Back-titration TSH Microspot Immunoassay employing Developing Antibody Conjugated to Fluorescent Microspheres

First Step

1) White polystyrene microtitre wells (Microlite 1 from Dynatech Laboratories) were spotted with 1µl or less of a 200µg/ml monoclonal anti-TSH capture antibody in 0.1M phosphate buffer at pH 7.4. The antibody droplets were aspirated immediately and the wells blocked with 1% (w/v) BSA and washed extensively with the same buffer. The antibody microspots were kept in buffer until use.

2) After rinsing with 0.05M/l Tris-HCl buffer at pH 7.75 (wash buffer), 200µl of either standard in assay buffer or the sample was added to each well and shaken for from 30 min to several hours at room temperature (or overnight at 4°C if maximal assay sensitivity is desired).

3) The wells were washed four times with wash buffer.

Second step

1) An aliquot of 200µl of developing binding material antibody conjugated to fluorescent-dye containing microspheres of diameter 0.1 µm (containing ~0.01 mg antibody-conjugated microspheres) in assay buffer was added to each well and shaken for 0.5 to 1 hour at room temperature.

2) The wells were washed seven times with the wash buffer which contained 0.05% Tween-20 (w/v), aspirated until completely dry and scanned with an MRC-600 Laser Scanning

arbitrary units against TSH concentration (x-axis) in mU/litre. The sensitivity of the assay (based on measurements of the standard deviation of the zero dose estimate) was 0.002 mU/litre. The same standards and assay buffer as those used in Example 5 were employed.

Example 7

Example 6 was repeated except that the total incubation time was reduced to 1 hour (0.5 hour incubation of sample with capture antibody, followed by 0.5 hour incubation with developing antibody) and the size of the microspheres was increased to 0.12 μm diameter. The sensitivity of the assay was thereby increased ten-fold to 0.0002 mU/litre, based on measurements of the standard deviation of the zero dose estimate. The results are plotted in Figure 7 of the accompany drawings, which is a graph on the same axes as Figure 6.

Example 8

A Single-step Ultra-sensitive Sandwich TSH Microspot Immunoassay Using Developing Antibody Conjugated to Fluorescent Microspheres

1) White polystyrene microtitre wells (Microlite 1 from Dynatech Laboratories) were spotted with 1μl or less of a 200μg/ml monoclonal anti-TSH capture antibody in 0.1 M phosphate buffer at pH 7.4. The antibody droplets were aspirated immediately and the wells blocked with 1% (w/v) BSA and washed extensively with the same buffer. The antibody microspots were kept in buffer until use.

2) The wells were rinsed with the assay buffer used in Example 5, then 100μl of standard in assay buffer/sample and 100μl of developing antibody-conjugated microspheres were added to each well and shaken at room temperature for 30 minutes, or longer if maximal assay sensitivity was desired.

orange/red dyes were scanned using the 488 and 568 nm lines of the krypton/argon mixed-gas laser. This could be done either simultaneously or consecutively. The concentration of antigen (TSH) in the test sample was obtained by observing the ratio of the fluorescent signals from the two dyes and correlating it with the signals using the standard samples.

The results obtained are shown in Figure 8 of the accompanying drawings, which is a graph of the ratio of the two fluorescent signals (y-axis) against TSH concentration (x-axis) in mU/litre. The sensitivity of the assay (based on measurements of the standard deviation of the zero dose estimate) was 0.0002 mU/litre.

Example 11

Single-labelled or Dual-labelled Ultra-sensitive Sandwich Single- or Two-step Back-titration TSH Microspot Immunoassay Using Biotinylated Developing antibody and a Universal Reagent of Avidin conjugated Fluorescent Microspheres

In contrast to the assay systems described in Examples 5 to 10, a universal marker reagent of avidin conjugated fluorescent microspheres was used in this Example to tag indirectly the bound developing antibody which had been labelled with biotin.

Although this assay system requires an additional step of the addition of avidin microspheres after the completion of the immunological reactions, the advantage of being able to use a "universal marker" outweighs this minor drawback. The "universal marker" system would be particularly useful in a microspot multianalyte system described by Roger Ekins in WO-89/01157 because of the considerable improvements in assay sensitivity that can be expected as a result of the reduction in non-specific binding from employing a single universal avidin-microsphere preparation rather than the

litre), 10mmol/l Tris-HCl pH7.5, and 1mmol/l EDTA. For a quantitative assay, the prepared samples or, as the case may be, standards containing single-stranded target DNA in known amounts and in the same hybridisation buffer were added to the wells (positive and negative controls rather than the standards were added to the other wells for the qualitative tests), incubated with shaking at 50°C for 1 hour and washed with PBS containing 0.05% Tween 20.

4) 200 µl of anti-double-stranded DNA antibody conjugated to fluorescent microspheres of diameter 0.1 µm (FluoSpheres) by the method described in Example 3 and in PBS containing 0.5% BSA and 0.05% Tween 20 was added, incubated with shaking for 1 hour at room temperature, washed with PBS-Tween 20 and scanned with the confocal microscope as described in Example 5.

Example 12b

Microspot sandwich DNA sequence assay using a biotinylated solid-phased capture DNA probe, a complementary but non-overlapping developing DNA probe labelled with digoxigenin and anti-digoxigenin antibody conjugated to microspheres containing fluorescent dye.

1) The avidin-biotinylated capture DNA probe microspots were prepared as described in Example 12a.

2) The hybridisation step was carried out as described in Example 12a.

3) 5 to 10 ng of the complementary but non-overlapping developing DNA probe labelled with digoxigenin (as described in the Nickerson et al reference above) in 100 µl of hybridization buffer was added, incubated with shaking at 50°C for 1 hour and washed with PBS-Tween 20.

4) 200 µl of the anti-digoxigenin antibody-conjugated

Tris-HCl pH 7.5, and 1 mmol/l EDTA. For a quantitative assay, the prepared sample plus the competitive material of target DNA sequences generated by polymerase chain reaction and labelled with fluorescent microspheres of diameter 0.1 μm (FluoSpheres) prepared using a technique modified from that described in the Wolf et al reference mentioned above for the attachment of DNA to latex particles, or standards containing single-stranded target DNA plus the competitive material and in the same hybridization buffer were added to the wells (positive and negative controls plus the competitive material instead of the standards plus the competitive material were added for the qualitative tests), incubated with shaking at 50°C for 1 hour, washed with Tris-HCl containing 0.05% Tween 20 and scanned as described in Example 5.

As indicated above, these very high sensitivities for non-competitive immunoassays are unexpected in the light of the currently accepted views on assay design. Some increase in sensitivity would be expected in any assay format, once the idea of using microspheres in accordance with the invention has been appreciated, because of the increased number of molecules of label attached to each molecule of developing binding material, this resulting in an effective increase in specific activity of the labelled developer molecules. However, this effect alone might not be expected to result in assay designs departing so markedly from conventional concepts in this field and requiring in particular very small amounts of capture binding agent.

Two further possible explanations for these unexpected findings can perhaps be advanced. The first is that by confining a very small number of capture binding agent molecules at high surface density to a very small area in the form of a microspot the signal/noise ratios obtained in any finite incubation time may be improved as compared with those obtained in conventional designs in which very large amounts of capture antibody are distributed over large

CLAIMS

1. A binding assay process in which the concentration of an analyte in a liquid sample is determined by comparison with a dose-response curve computed from standard samples,

using a capture binding agent having binding sites specific for the analyte and a developing binding material capable of binding with the bound analyte or with the binding sites on the capture binding agent occupied by the bound analyte or with the binding sites remaining unoccupied on the capture binding agent,

the capture binding agent being used in an amount such that only an insignificant fraction of the analyte in the sample becomes bound to the capture binding agent, and

a label being used in the assay in relation to the developing binding material whereby the strength of the signal associated with the label is representative of the fractional occupancy of the binding sites on the capture binding agent by the analyte,

wherein there is used as the label microspheres having a size of less than 5 μm and carrying a marker.

2. A process as claimed in claim 1, wherein the microspheres have a uniform size of 0.01 to 0.5 μm.

3. A process as claimed in claim 1 or 2, wherein the microspheres are made of polymer latex and are provided on their surface with negatively charged or positively charged groups.

4. A process as claimed in any of claims 1 to 3, wherein the marker is a fluorescent label contained within the microspheres.

5. A process as claimed in claim 4, wherein the microspheres contain molecules of an oil-soluble fluorescent dye providing fluorescence in a colour range compatible with

wherein the capture binding agent is immobilised on a solid support in the form of one or more microspots having an area of 1 mm^2 or less at a surface density in the range of 1,000 to 100,000 molecules/ μm^2, and wherein the liquid sample size is 1 ml or less.

12. A process as claimed in claim 11, wherein the microspot or microspots have a diameter of 0.01 to 1 mm and contain immobilised capture binding agent at a surface density of 10,000 to 50,000 molecules/ μm^2, the sample size being 50 μl - 1 ml.

13. A process as claimed in claim 11 or 12, wherein different capture binding agents are immobilised on different microspots on the same solid support and different binding assays for the determination of different analytes in the same liquid sample are performed in the same operation.

14. A process as claimed in any of claims 1 to 13, wherein both the capture binding agent and the developing binding material are antibodies.

15. A process as claimed in any of claims 1 to 13, for use in DNA assays, wherein the capture binding agent is a single-stranded oligonucleotide DNA probe recognising a corresponding DNA sequence in the liquid sample and the developing binding material either is an antibody recognising only twin-stranded DNA sequences or is an oligonucleotide DNA sequence which either recognises another part of the corresponding DNA sequence in the liquid sample or recognises residual single-stranded oligonucleotide DNA probe forming the capture binding agent, the developing binding material being labelled by means of the microspheres.

16. A process as claimed in any of claims 1 to 15, wherein the binding assay is a non-competitive binding

sites specific for the analyte and a developing binding material capable of binding with the bound analyte or with the binding sites on the capture binding agent occupied by the bound analyte or with the binding sites remaining unoccupied on the capture binding agent, a label being used in relation to the developing binding material whereby the strength of the signal associated with the label is representative of the fractional occupancy of the binding sites on the capture binding agent by the analyte, the kit comprising (a) a solid support having the capture binding agent immobilised thereon, (b) a developing reagent comprising the developing binding material adsorbed or directly or indirectly chemically bonded to the surface of microspheres carrying a marker and (c) standards having known amounts or concentrations of the analyte to be determined.

21. A kit as claimed in claim 20, wherein the reagent contains the developing binding material adsorbed onto or covalently bonded to microspheres having a size of less than 5 μm and containing molecules of a fluorescent dye.

22. A kit as claimed in claim 20 or 21, wherein the solid support has the capture binding agent immobilised thereon in the form of one or more microspots of size less than 1 mm^2 and surface density at least 1000 molecules/ μm^2.

23. A kit as claimed in claim 22, wherein different capture binding agents are immobilised on different microspots on the same solid support and a plurality of different developing reagents and different standards for different analytes are included.

FIG.3

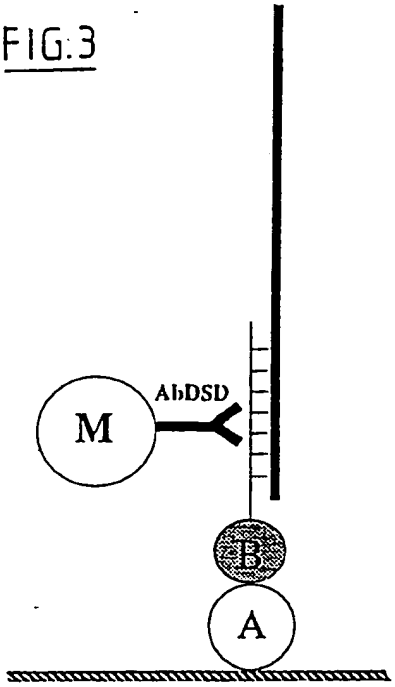


FIG. 4

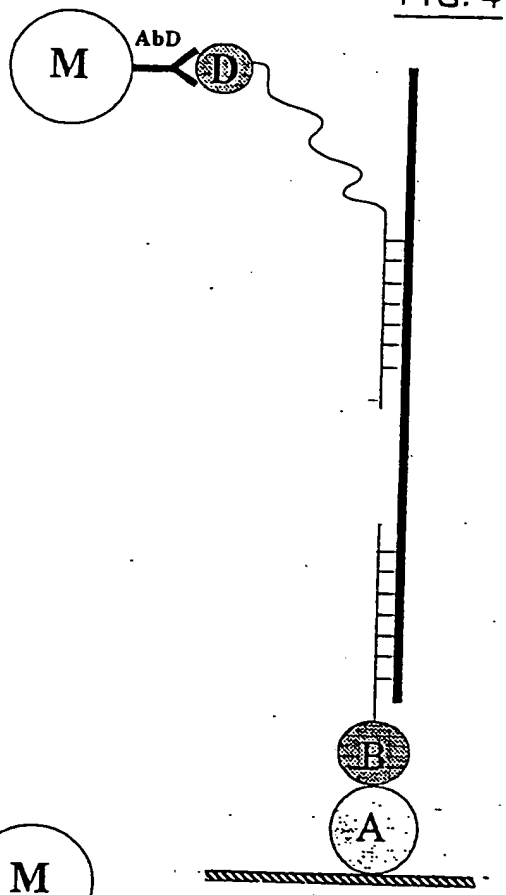


FIG.5

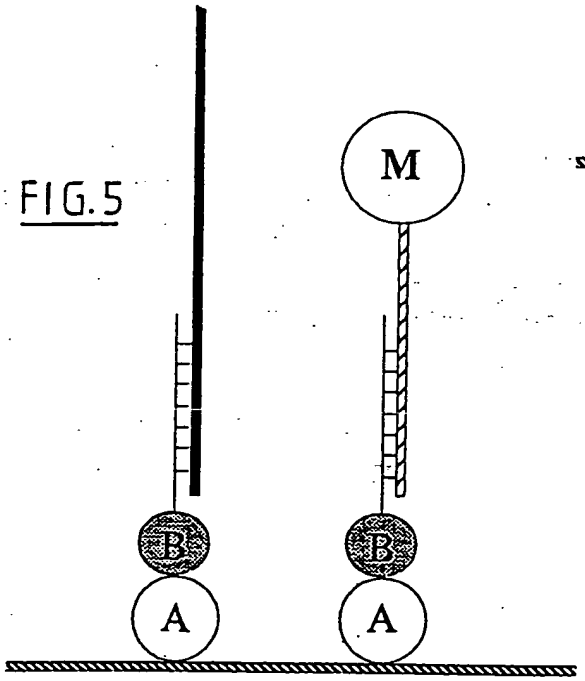
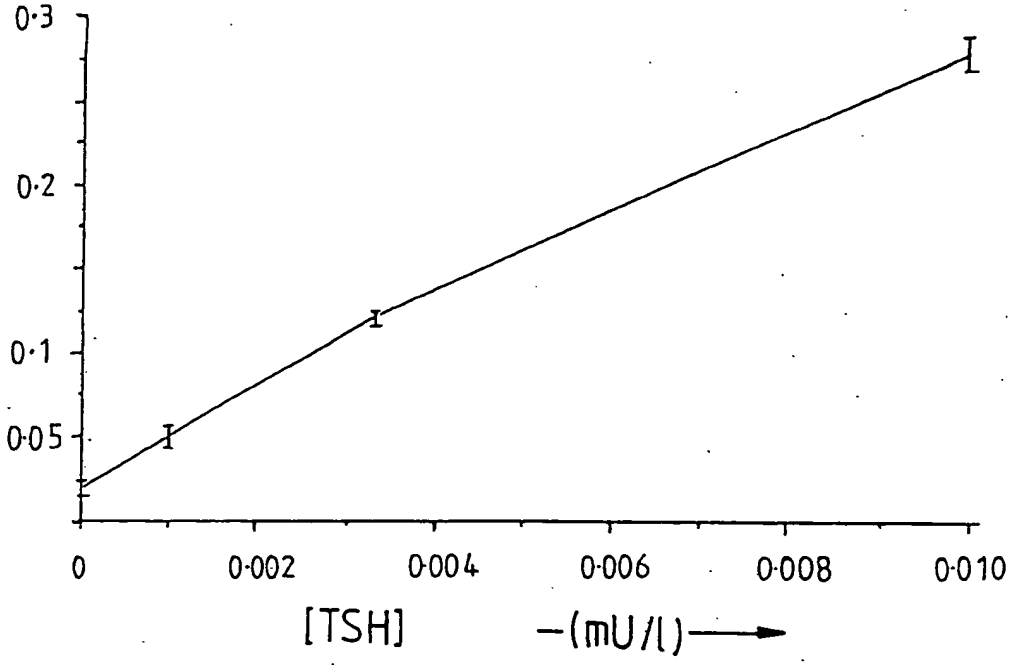


FIG. 8



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/01892

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 G01N33/543; G01N33/58; C12Q1/68

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	G01N ; C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[10]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP,A,0 396 801 (TEIKOKU HORMONE MFG CO.,LTD) 14 November 1990 see the whole document	1-23
X	EP,A,0 360 088 (ABBOTT LABORATORIES) 28 March 1990 see page 3, line 40 - line 50 see page 6, line 1 - line 40; claims 10,11	1-23
X	EP,A,0 301 584 (FUJIREBIO K.K) 1 February 1989 see the whole document	1-23
X	EP,A,0 267 317 (PROFILE DIAGNOSTIC SCIENCES INC.) 18 May 1988 see claims 1,3	1-23

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[10] Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29 JANUARY 1993	17.02.93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	CARTAGENA ABELLA P

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9201892 SA 65784

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 29/01/93

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	WO,A,8 801 058 (R. PH. EKINS) 11 February 1988 cited in the application	
A	WO,A,8 901 157 (R. PH. EKINS) 9 February 1989 cited in the application	
A	WO,A,8 401 031 (R. PH. EKINS) 15 March 1984 cited in the application	